PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12Q 1/68 — A2

(11) International Publication Number: **WO 99/66075**

(43) International Publication Date: 23 December 1999 (23.12.99)

(21) International Application Number: PCT/US99/13426

(22) International Filing Date: 14 June 1999 (14.06.99)

(30) Priority Data:

09/098,079	15 June 1998 (15.06.98)	US
09/097,889	15 June 1998 (15.06.98)	US
09/302,681	30 April 1999 (30.04.99)	US

(71) Applicant *(for all designated States except US)*: MITOKOR [US/US]; 11494 Sorrento Valley Road, San Diego, CA 92121 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: HERRNSTADT, Corinna [DE/US]; 3628 Ruette DeVille, San Diego, CA 92130 (US). GHOSH, Soumitra, S. [US/US]; 12334 Pathos Lane, San Diego, CA 92129 (US). CLEVENGER, William [US/US]; 1515 South Melrose Drive #43, Vista, CA 92083 (US). FAHY, Eoin, D. [US/US]; 7171 Canon Hill Way, San Diego, CA 92126 (US). DAVIS, Robert, E. [US/US]; 13272 Glencliff Way, San Diego, CA 92130 (US).

(74) Agents: ROSENMAN, Stephen, J. et al.; Seed and Berry LLP, 6300 Columbia Center, 701 Fifth Avenue, Seattle, WA 98104–7092 (US).

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published

Without international search report and to be republished upon receipt of that report.

(54) Title: DIAGNOSTIC METHOD BASED ON QUANTIFICATION OF EXTRAMITOCHONDRIAL DNA

(57) Abstract

Compositions and methods based on quantification of extramitochondrial DNA (exmtDNA) sequences are provided that are useful for detecting the presence of or risk for having a disease associated with altered mitochondrial function, and for identifying agents suitable for treating such diseases. The exmtDNA sequences have strong homology to authentic mitochondrial DNA (mtDNA) sequences.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	**ES**	Spain	**LS**	Lesotho	**SI**	Slovenia
AM	Armenia	**FI**	Finland	**LT**	Lithuania	**SK**	Slovakia
AT	Austria	**FR**	France	**LU**	Luxembourg	**SN**	Senegal
AU	Australia	**GA**	Gabon	**LV**	Latvia	**SZ**	Swaziland
AZ	Azerbaijan	**GB**	United Kingdom	**MC**	Monaco	**TD**	Chad
BA	Bosnia and Herzegovina	**GE**	Georgia	**MD**	Republic of Moldova	**TG**	Togo
BB	Barbados	**GH**	Ghana	**MG**	Madagascar	**TJ**	Tajikistan
BE	Belgium	**GN**	Guinea	**MK**	The former Yugoslav Republic of Macedonia	**TM**	Turkmenistan
BF	Burkina Faso	**GR**	Greece			**TR**	Turkey
BG	Bulgaria	**HU**	Hungary	**ML**	Mali	**TT**	Trinidad and Tobago
BJ	Benin	**IE**	Ireland	**MN**	Mongolia	**UA**	Ukraine
BR	Brazil	**IL**	Israel	**MR**	Mauritania	**UG**	Uganda
BY	Belarus	**IS**	Iceland	**MW**	Malawi	**US**	United States of America
CA	Canada	**IT**	Italy	**MX**	Mexico	**UZ**	Uzbekistan
CF	Central African Republic	**JP**	Japan	**NE**	Niger	**VN**	Viet Nam
CG	Congo	**KE**	Kenya	**NL**	Netherlands	**YU**	Yugoslavia
CH	Switzerland	**KG**	Kyrgyzstan	**NO**	Norway	**ZW**	Zimbabwe
CI	Côte d'Ivoire	**KP**	Democratic People's Republic of Korea	**NZ**	New Zealand		
CM	Cameroon			**PL**	Poland		
CN	China	**KR**	Republic of Korea	**PT**	Portugal		
CU	Cuba	**KZ**	Kazakstan	**RO**	Romania		
CZ	Czech Republic	**LC**	Saint Lucia	**RU**	Russian Federation		
DE	Germany	**LI**	Liechtenstein	**SD**	Sudan		
DK	Denmark	**LK**	Sri Lanka	**SE**	Sweden		
EE	Estonia	**LR**	Liberia	**SG**	Singapore		

DIAGNOSTIC METHOD BASED ON QUANTIFICATION OF EXTRAMITOCHONDRIAL DNA

TECHNICAL FIELD

The present invention relates generally to diseases in which altered mitochondrial function, such as free radical mediated oxidative injury, leads to tissue degeneration and, more specifically, to compositions and methods for detecting predisposition to such diseases by quantifying extramitochondrial DNA.

BACKGROUND OF THE INVENTION

A number of degenerative diseases are thought to be caused by or be associated with alterations in mitochondrial function. These diseases include Alzheimer's Disease, diabetes mellitus, Parkinson's Disease, Huntington's disease, dystonia, Leber's hereditary optic neuropathy, schizophrenia, and myodegenerative disorders such as "mitochondrial encephalopathy, lactic acidosis, and stroke" (MELAS), and "myoclonic epilepsy ragged red fiber syndrome" (MERRF). Other diseases involving altered metabolism or respiration within cells may also be regarded as diseases associated with altered mitochondrial function.

Functional mitochondria contain gene products encoded by mitochondrial genes situated in mitochondrial DNA (mtDNA) and by extramitochondrial genes not situated in the circular mitochondrial genome. The 16.5 kb mtDNA encodes 22 tRNAs, two ribosomal RNAs (rRNA) and only 13 enzymes of the electron transport chain (ETC), the elaborate multi-complex mitochondrial assembly where, for example, respiratory oxidative phosphorylation takes place. The overwhelming majority of mitochondrial structural and functional proteins are encoded by extramitochondrial, and in most cases presumably nuclear, genes. Accordingly, mitochondrial and extramitochondrial genes may interact directly, or indirectly via gene products and their downstream intermediates, including metabolites, catabolites, substrates, precursors, cofactors and the like. Alterations in mitochondrial function, for example impaired electron transport activity, defective oxidative phosphorylation or

increased free radical production, may therefore arise as the result of defective mtDNA, defective extramitochondrial DNA, defective mitochondrial or extramitochondrial gene products, defective downstream intermediates or a combination of these and other factors.

Mitochondria are the subcellular organelles that manufacture bioenergetically essential adenosine triphosphate (ATP) by oxidative phosphorylation. Defective mitochondrial activity, including failure at any step of the ETC, may result in the generation of highly reactive free radicals that have the potential of damaging cells and tissues. These free radicals may include reactive oxygen species (ROS) such as superoxide, peroxynitrite and hydroxyl radicals, and potentially other reactive species that may be toxic to cells. For example, oxygen free radical induced lipid peroxidation is a well established pathogenetic mechanism in central nervous system (CNS) injury, such as that found in a number of degenerative diseases, and in ischemia (*i.e.*, stroke).

There are at least two deleterious consequences of exposure to reactive free radicals arising from mitochondrial dysfunction that adversely impact the mitochondria themselves. First, free radical mediated damage may inactivate one or more of the myriad proteins of the ETC. According to generally accepted theories of mitochondrial function, proper ETC respiratory activity requires maintenance of an electrochemical potential in the inner mitochondrial membrane by a coupled chemiosmotic mechanism. Free radical oxidative activity may dissipate this membrane potential, thereby preventing ATP biosynthesis and halting the production of a vital biochemical energy source. In addition, mitochondrial proteins such as cytochrome c and "apoptosis inducing factor" may leak out of the mitochondria after permeability transition and may induce the genetically programmed cell suicide sequence known as apoptosis or programmed cell death (PCD).

Second, free radical mediated damage may result in catastrophic mitochondrial collapse that has been termed "transition permeability". For example, rapid mitochondrial permeability transition likely entails changes in the inner mitochondrial transmembrane protein adenylate translocase that results in the formation of a "pore." In any event, because permeability transition is potentiated by free radical

exposure. it may be more likely to occur in the mitochondria of cells from patients having mitochondria associated diseases that are chronically exposed to such reactive free radicals.

Altered mitochondrial function characteristic of the mitochondria associated diseases may also be related to loss of mitochondrial membrane electrochemical potential by mechanisms other than free radical oxidation, and such transition permeability may result from direct or indirect effects of mitochondrial genes, gene products or related downstream mediator molecules and/or extramitochondrial genes. gene products or related downstream mediators, or from other known or unknown causes.

Diabetes mellitus is a common, degenerative disease affecting 5 to 10 percent of the population in developed countries. The propensity for developing diabetes mellitus is reportedly maternally inherited, suggesting a mitochondrial genetic involvement. (Alcolado, J.C. and Alcolado, R., *Br. Med. J. 302*:1178-1180 (1991); Reny. S.L., *International J. Epidem. 23*:886-890 (1994)). Diabetes is a heterogenous disorder with a strong genetic component; monozygotic twins are highly concordant and there is a high incidence of the disease among first degree relatives of affected individuals.

At the cellular level, the degenerative phenotype that may be characteristic of late onset diabetes mellitus includes indicators of altered mitochondrial respiratory function, for example impaired insulin secretion, decreased ATP synthesis and increased levels of reactive oxygen species. Studies have shown that diabetes mellitus may be preceded by or associated with certain related disorders. For example, it is estimated that forty million individuals in the U.S. suffer from late onset impaired glucose tolerance (IGT). IGT patients fail to respond to glucose with increased insulin secretion. A small percentage of IGT individuals (5-10%) progress to insulin deficient non-insulin dependent diabetes (NIDDM) each year. Some of these individuals further progress to insulin dependent diabetes mellitus (IDDM). These forms of diabetes mellitus. NIDDM and IDDM, are associated with decreased release of insulin by pancreatic beta cells and/or a decreased end-organ response to insulin. Other symptoms

of diabetes mellitus and conditions that precede or are associated with diabetes mellitus include obesity, vascular pathologies, peripheral and sensory neuropathies, blindness and deafness.

Parkinson's disease (PD) is a progressive, neurodegenerative disorder associated with altered mitochondrial function and characterized by the loss and/or atrophy of dopamine-containing neurons in the *pars compacta* of the *substantia nigra* of the brain. Like Alzheimer's Disease (AD), PD also afflicts the elderly. It is characterized by bradykinesia (slow movement), rigidity and a resting tremor. Although L-Dopa treatment reduces tremors in most patients for a while, ultimately the tremors become more and more uncontrollable, making it difficult or impossible for patients to even feed themselves or meet their own basic hygiene needs.

It has been shown that the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) induces parkinsonism in animals and man at least in part through its effects on mitochondria. MPTP is converted to its active metabolite, MPP+, in dopamine neurons; it then becomes concentrated in the mitochondria. The MPP+ then selectively inhibits the mitochondrial enzyme NADH:ubiquinone oxidoreductase ("Complex I"), leading to the increased production of free radicals, reduced production of adenosine triphosphate, and ultimately, the death of affected dopamine neurons.

Mitochondrial Complex I is composed of 40-50 subunits; most are encoded by the nuclear genome and seven by the mitochondrial genome. Since parkinsonism may be induced by exposure to mitochondrial toxins that affect Complex I activity, it appears likely that defects in Complex I proteins may contribute to the pathogenesis of PD by causing a similar biochemical deficiency in Complex I activity. Indeed, defects in mitochondrial Complex I activity have been reported in the blood and brain of PD patients (Parker et al., *Am. J. Neurol. 26*:719-723, 1989).

Alzheimer's disease (AD) is a progressive neurodegenerative disorder that is characterized by loss and/or atrophy of neurons in discrete regions of the brain, and that is accompanied by extracellular deposits of β-amyloid and the intracellular accumulation of neurofibrillary tangles. It is a uniquely human disease, affecting over 13 million people worldwide. It is also a uniquely tragic disease. Many individuals

who have lived normal, productive lives are slowly stricken with AD as they grow older, and the disease gradually robs them of their memory and other mental faculties. Eventually, they cease to recognize family and loved ones, and they often require continuous care until their eventual death.

There is evidence that defects in oxidative phosphorylation within the mitochondria are at least a partial cause of sporadic AD. The enzyme cytochrome c oxidase (COX), which makes up part of the mitochondrial electron transport chain (ETC), is present in normal amounts in AD patients; however, the catalytic activity of this enzyme in AD patients and in the brains of AD patients at autopsy has been found to be abnormally low. This suggests that the COX in AD patients is defective, leading to decreased catalytic activity that in some fashion causes or contributes to the symptoms that are characteristic of AD.

Focal defects in energy metabolism in the mitochondria, with accompanying increases in oxidative stress, may be associated with AD. It is well-established that energy metabolism is impaired in AD brain (Palmer et al., *Brain Res. 645*:338-42, 1994; Pappolla et al., *Am. J. Pathol. 140*:621-28, 1992; Jeandel et al., *Gerontol. 35*:275, 1989; Balazs et al., *Neurochem. Res. 19*:1131-37, 1994; Mecocci et al., *Ann. Neurol. 36*:747-751, 1994; Gsell et al., *J. Neurochem. 64*:1216-23, 1995). For example, regionally specific deficits in energy metabolism in AD brains have been reported in a number of positron emission tomography studies (Kuhl, et al., *J. Cereb. Blood Flow Metab. 7*:S406, 1987; Grady, et al., *J. Clin. Exp. Neuropsychol. 10*:576-96, 1988; Haxby et al., *Arch. Neurol. 47*:753-60, 1990; Azari et al., *J. Cereb. Blood Flow Metab. 13*:438-47, 1993). Metabolic defects in the temporoparietal neocortex of AD patients apparently presage cognitive decline by several years. Skin fibroblasts from AD patients display decreased glucose utilization and increased oxidation of glucose, leading to the formation of glycosylation end products (Yan et al., *Proc. Nat. Acad. Sci. USA 91*:7787-91, 1994). Cortical tissue from postmortem AD brain shows decreased activity of the mitochondrial enzymes pyruvate dehydrogenase (Sheu et al., *Ann. Neurol. 17*:444-49, 1985) and α–ketoglutarate dehydrogenase (Mastrogiacomo et al., *J. Neurochem. 6*:2007-14, 1994), which are both key enzymes in energy metabolism.

Functional magnetic resonance spectroscopy studies have shown increased levels of inorganic phosphate relative to phosphocreatine in AD brain, suggesting an accumulation of precursors that arises from decreased ATP production by mitochondria (Pettegrew et al., *Neurobiol. of Aging 15*:117-32, 1994; Pettigrew et al., *Neurobiol. of Aging 16*:973-75, 1995). In addition, the levels of pyruvate, but not of glucose or lactate, are reported to be increased in the cerebrospinal fluid of AD patients, consistent with defects in cerebral mitochondrial electron transport chain (ETC) activity (Parnetti et al., *Neurosci. Lett. 199*:231-33, 1995).

Signs of oxidative injury are prominent features of AD pathology and, as noted above, reactive oxygen species (ROS) are critical mediators of neuronal degeneration. Indeed, studies at autopsy show that markers of protein, DNA and lipid peroxidation are increased in AD brain (Palmer et al., Brain Res. 645:338-42, 1994; Pappolla et al., Am. J. Pathol. 140:621-28, 1992; Jeandel et al., Gerontol. 35:275-82, 1989; Balazs et al., Arch. Neurol. 4:864, 1994; Mecocci et al., Ann. Neurol. 36:747-51, 1994; Smith et al., Proc. Nat. Acad. Sci. USA 88:10540-43, 1991). In hippocampal tissue from AD but not from controls, carbonyl formation indicative of protein oxidation is increased in neuronal cytoplasm, and nuclei of neurons and glia (Smith et al., *Nature 382*:120-21, 1996). Neurofibrillary tangles also appear to be prominent sites of protein oxidation (Schweers et al., *Proc. Nat. Acad. Sci. USA 92*:8463, 1995; Blass et al., *Arch. Neurol. 4*:864, 1990). Under stressed and non-stressed conditions incubation of cortical tissue from AD brains taken at autopsy demonstrate increased free radical production relative to non-AD controls. In addition, the activities of critical antioxidant enzymes, particularly catalase, are reduced in AD (Gsell et al., *J. Neurochem. 64*:1216-23, 1995), suggesting that the AD brain is vulnerable to increased ROS production. Thus, oxidative stress may contribute significantly to the pathology of mitochondria associated diseases such as AD, where mitochondrial dysfunction and/or elevated ROS may be present.

One hallmark pathology of AD is the death of selected neuronal populations in discrete regions of the brain. Cell death in AD is presumed to be apoptotic because signs of programmed cell death (PCD) are seen and indicators of

active gliosis and necrosis are not found. (Smale et al., *Exp. Neurolog. 133*:225-230, 1995; Cotman et al., *Molec. Neurobiol. 10*:19-45, 1995.) The consequences of cell death in AD, neuronal and synaptic loss, are closely associated with the clinical diagnosis of AD and are highly correlated with the degree of dementia in AD (DeKosky et al., *Ann. Neurology 27*:457-464, 1990).

Mitochondrial dysfunction is thought to be critical in the cascade of events leading to apoptosis in various cell types (Kroemer et al., *FASEB J. 9*:1277-87, 1995), and may be a cause of apoptotic cell death in neurons of the AD brain. Altered mitochondrial physiology may be among the earliest events in PCD (Zamzami et al., *J. Exp. Med. 182*:367-77, 1995; Zamzami et al., *J. Exp. Med. 181*:1661-72, 1995) and elevated reactive oxygen species (ROS) levels that result from such altered mitochondrial function may initiate the apoptotic cascade (Ausserer et al., *Mol. Cell. Biol. 14*:5032-42, 1994). In several cell types, including neurons, reduction in the mitochondrial membrane potential ($\Delta\Psi m$) precedes the nuclear DNA degradation that accompanies apoptosis. In cell-free systems, mitochondrial, but not nuclear, enriched fractions are capable of inducing nuclear apoptosis (Newmeyer et al., *Cell 70*:353-64, 1994). Perturbation of mitochondrial respiratory activity leading to altered cellular metabolic states, such as elevated intracellular ROS, may occur in mitochondria associated diseases and may further induce pathogenetic events via apoptotic mechanisms.

Oxidatively stressed mitochondria may release a pre-formed soluble factor that can induce chromosomal condensation, an event preceding apoptosis (Marchetti et al., *Cancer Res. 56*:2033-38, 1996). In addition, members of the Bcl-2 family of anti-apoptosis gene products are located within the outer mitochondrial membrane (Monaghan et al., *J. Histochem. Cytochem. 40*:1819-25, 1992) and these proteins appear to protect membranes from oxidative stress (Korsmeyer et al, *Biochim. Biophys. Act. 1271*:63, 1995). Localization of Bcl-2 to this membrane appears to be indispensable for modulation of apoptosis (Nguyen et al., *J. Biol. Chem. 269*:16521-24, 1994). Thus, changes in mitochondrial physiology may be important mediators of apoptosis. To the extent that apoptotic cell death is a prominent feature of neuronal loss

in AD. mitochondrial dysfunction may be critical to the progression of this disease and may also be a contributing factor in other mitochondria associated diseases.

Regardless of whether a defect underlying a disease associated with altered mitochondrial function may have mitochondrial or extramitochondrial origins, and regardless of whether a defect underlying altered mitochondrial function has been identified, the present invention provides methods that are useful for determining the risk or presence of diseases associated with such altered mitochondrial function, and for identifying agents that are suitable for treating such diseases. In particular, as is elaborated herein below, the present invention provides compositions and methods for the detection of diseases associated with altered mitochondrial function by quantification of unusual mtDNA-like sequences not found in mitochondria and referred to as extramitochondrial DNA (exmtDNA), and other related advantages.

SUMMARY OF THE INVENTION

Briefly stated, the present invention is directed to compositions and methods useful for detecting mitochondria associated diseases and involving extramitochondrial DNA (exmtDNA) sequences that are highly homologous to mitochondrial DNA (mtDNA). In one aspect the invention provides a method for determining the risk for or presence of a disease associated with altered mitochondrial function in a first subject suspected of having or being at risk for having such a disease, by comparing a ratio r for each of a first and a second biological sample containing extramitochondrial DNA and mitochondrial DNA, the first biological sample being obtained from the first subject and the second sample being obtained from a second subject known to be free of a risk or presence of a disease associated with altered mitochondrial function, using the formula:

$$r = x/(x + y)$$

wherein x is the amount of extramitochondrial DNA in a sample, and y is the amount of mitochondrial DNA in the sample; and therefrom determining the risk or presence of the disease. In an embodiment of the invention, the ratio r is calculated by a method

that comprises contacting a biological sample containing extramitochondrial DNA and mitochondrial DNA with an oligonucleotide primer having a nucleotide sequence that is complementary to a sequence present in the extramitochondrial DNA and present in the mitochondrial DNA, under conditions and for a time sufficient to allow hybridization of the primer to the extramitochondrial DNA and to the mitochondrial DNA; and detecting hybridization of the primer to the extramitochondrial DNA and to the mitochondrial DNA, in order to therefrom quantify the extramitochondrial DNA and the mitochondrial DNA.

In another embodiment, the ratio *r* is calculated by a method comprising contacting a sample containing amplified extramitochondrial DNA and mitochondrial DNA with an oligonucleotide primer having a nucleotide sequence that is complementary to a sequence present in the amplified extramitochondrial DNA and present in the amplified mitochondrial DNA, under conditions and for a time sufficient to allow hybridization of the primer to the extramitochondrial DNA and to the mitochondrial DNA; and detecting hybridization of the primer to the extramitochondrial DNA and to the mitochondrial DNA, and therefrom quantifying the extramitochondrial DNA and the mitochondrial DNA. In another embodiment of this aspect of the invention the ratio *r* is calculated by a method comprising contacting a biological sample containing extramitochondrial DNA and mitochondrial DNA with an oligonucleotide primer having a nucleotide sequence that is complementary to a sequence present in the extramitochondrial DNA and present in the mitochondrial DNA, under conditions and for a time sufficient to allow hybridization of said primer to the extramitochondrial DNA and to the mitochondrial DNA; and detecting hybridization and extension of the primer to the extramitochondrial DNA to produce a first product and hybridization and extension of the primer to the mitochondrial DNA to produce a second product distinguishable from the first product, and therefrom quantifying the extramitochondrial DNA and the mitochondrial DNA.

In another embodiment of this aspect of the invention the ratio *r* is calculated by a method comprising contacting a sample containing amplified extramitochondrial DNA and mitochondrial DNA with an oligonucleotide primer

having a nucleotide sequence that is complementary to a sequence present in the amplified extramitochondrial DNA and present in the amplified mitochondrial DNA, under conditions and for a time sufficient to allow hybridization of the primer to the extramitochondrial DNA and to the mitochondrial DNA; and detecting hybridization and extension of the primer to the extramitochondrial DNA to produce a first product and hybridization and extension of the primer to the mitochondrial DNA to produce a second product distinguishable from the first product, and therefrom quantifying the extramitochondrial DNA and the mitochondrial DNA.

In another embodiment of this aspect of the invention the biological sample is treated by heating it in water to lyse cells contained in the sample, and then extracting cellular DNA from the lysed cells using an aqueous DNA extraction procedure. In certain embodiments of the invention the sample comprises a crude buffy coat fraction of whole blood. In certain other embodiments of the invention, the method further comprises the step of determining the ApoE genotype of the first subject and correlating said genotype with the risk or presence of disease. In some embodiments of the invention, the disease associated with altered mitochondrial function may be Alzheimer's Disease, Huntington's Disease, Parkinson's Disease, dystonia, schizophrenia, non-insulin dependent diabetes mellitus, mitochondrial encephalopathy, lactic acidosis, and stroke, myoclonic epilepsy ragged red fiber syndrome, and Leber's hereditary optic neuropathy.

Another aspect of the invention provides a method for quantifying extramitochondrial DNA, comprising: contacting a sample containing extramitochondrial DNA with an oligonucleotide primer having a nucleotide sequence complementary to at least a portion of the extramitochondrial DNA under conditions and for a time sufficient to allow hybridization of the primer to the extramitochondrial DNA; and detecting hybridization of the primer to the extramitochondrial DNA, and therefrom quantifying the extramitochondrial DNA.

It is another aspect of the invention to provide a method for quantifying extramitochondrial DNA, comprising: contacting a sample containing extramitochondrial DNA with an oligonucleotide primer having a nucleotide sequence

complementary to at least a portion of the extramitochondrial DNA under conditions and for a time sufficient to allow hybridization of the primer to the extramitochondrial DNA: and detecting hybridization and extension of the primer to the extramitochondrial DNA to produce a product. and therefrom quantifying the extramitochondrial DNA.

Another aspect of the invention provides a method for quantifying extramitochondrial DNA. comprising: contacting a sample containing amplified extramitochondrial DNA with an oligonucleotide primer having a nucleotide sequence complementary to at least a portion of the extramitochondrial DNA under conditions and for a time sufficient to allow hybridization of the primer to the extramitochondrial DNA; and detecting hybridization of the primer to the extramitochondrial DNA, therefrom quantifying the extramitochondrial DNA.

In yet another aspect of the invention, a method is provided for quantifying extramitochondrial DNA by contacting a sample containing amplified extramitochondrial DNA with an oligonucleotide primer having a nucleotide sequence complementary to at least a portion of the extramitochondrial DNA under conditions and for a time sufficient to allow hybridization of the primer to the extramitochondrial DNA; and detecting hybridization and extension of the primer to the extramitochondrial DNA to produce a product, and therefrom quantifying the extramitochondrial DNA.

In one embodiment the extramitochondrial DNA is amplified by polymerase chain reaction, transcriptional amplification systems or self-sustained sequence replication. In certain embodiments of the various aspects of the invention, a single oligonucleotide primer is used. In certain embodiments of the invention a primer extension assay is used. In certain embodiments of the invention. the step of detecting may be by polymerase chain reaction, primer extension assay. ligase chain reaction or restriction fragment length polymorphism analysis.

In certain embodiments of the invention. the amount of extramitochondrial DNA in a biological sample is quantified by determining the presence in the sample of a nucleotide sequence that may be SEQ ID NO:1, a portion of SEQ ID NO:1, SEQ ID NO:3. a portion of SEQ ID NO:3. an extramitochondrial DNA sequence comprising a nucleic acid sequence that (i) corresponds to at least a portion of

SEQ ID NO:2 and (ii) contains at least one nucleotide substitution of Figure 4 at a corresponding nucleotide position, or an extramitochondrial DNA sequence comprising a nucleic acid sequence that (i) corresponds to at least a portion of SEQ ID NO:2 and (ii) contains at least one nucleotide deletion of Figure 4 at a corresponding nucleotide position. In one embodiment the portion of the nucleotide sequence of SEQ ID NO:1 corresponds to a portion of the nucleotide sequence of SEQ ID NO:2 encoding a mitochondrial cytochrome c oxidase. In another embodiment the portion of SEQ ID NO:1 corresponds to a portion of a mitochondrial cytochrome c oxidase encoding sequence that may be portion of a cytochrome c oxidase 1 (CO1) encoding sequence or a portion of a cytochrome c oxidase 2 (CO2) encoding sequence. In still other embodiments, the portion of the nucleotide sequence of SEQ ID NO:1 corresponds to a portion of the nucleotide sequence of SEQ ID NO:2 encoding a mitochondrial ATP synthetase subunit. In other embodiments, the portion of SEQ ID NO:1 corresponds to a portion of a mitochondrial ATP synthetase subunit encoding sequence that may be a portion of a sequence encoding ATP synthetase subunit 6 or a portion of a sequence encoding ATP synthetase subunit 8.

In some embodiments the nucleotide sequence of SEQ ID NO:1 corresponds to a portion of SEQ ID NO:2 that may be a portion of a sequence encoding ND1, a sequence encoding a portion of ND2 or a sequence encoding a portion of CO3. In other embodiments, the portion of the nucleotide sequence of SEQ ID NO:3 corresponds to a portion of the nucleotide sequence of SEQ ID NO:2 encoding a mitochondrial ATP synthetase subunit, which in some embodiments may further be a portion of a sequence encoding ATP synthetase subunit 6 or a portion of a sequence encoding ATP synthetase subunit 8. In still other embodiments, the nucleotide sequence of SEQ ID NO:1 corresponds to a portion of the nucleotide sequence of SEQ ID NO:2 encoding a mitochondrial tRNA, while in yet other embodiments the portion of the nucleotide sequence of SEQ ID NO:3 corresponds to a portion of the nucleotide sequence of SEQ ID NO:2 encoding a mitochondrial tRNA.

In another aspect the invention provides an isolated nucleic acid comprising all or a portion of the nucleotide sequence of SEQ ID NO:1 or a

complementary sequence thereto. In another aspect the invention provides an isolated nucleic acid comprising all or a portion of a nucleotide sequence of SEQ ID NO:1 or a complementary sequence thereto, wherein the sequence of the isolated nucleic acid differs by at least one nucleotide from the corresponding sequence of a nucleic acid comprising the nucleotide sequence of SEQ ID NO:2 or a complementary sequence thereto. In another aspect the invention provides an isolated nucleic acid comprising all or a portion of the nucleotide sequence of SEQ ID NO:3 or a complementary sequence thereto. In another aspect the invention provides an isolated nucleic acid comprising all or a portion of a nucleotide sequence of SEQ ID NO:3 or a complementary sequence thereto, wherein the sequence of the isolated nucleic acid differs by at least one nucleotide from the corresponding sequence of a nucleic acid comprising the nucleotide sequence of SEQ ID NO:2 or a complementary sequence thereto.

In another aspect the invention provides a method for determining the risk or presence of a disease associated with altered mitochondrial function in a subject suspected of having or being at risk for having such a disease, by quantifying the amount of extramitochondrial DNA and the amount of mitochondrial DNA in a biological sample from the subject, and therefrom determining the risk or presence of the disease. It is another aspect of the invention to provide a method for determining the risk or presence of a disease associated with altered mitochondrial function in a first subject suspected of having or being at risk for having such a disease, by comparing the amount of extramitochondrial DNA and the amount of mitochondrial DNA in a biological sample from the first subject to the amount of extramitochondrial DNA and the amount of mitochondrial DNA in a biological sample from a second subject, and therefrom determining the risk or presence of the disease. In another aspect the invention provides a method for determining the risk or presence of a disease associated with altered mitochondrial function in a first subject suspected of having or being at risk for having such a disease, by quantifying the amount of extramitochondrial DNA and the amount of mitochondrial DNA in a biological sample from the subject and comparing the amount of extramitochondrial DNA and the amount of mitochondrial DNA to the amount of extramitochondrial DNA and the amount of mitochondrial DNA

in a biological sample from a second subject known to be free of a risk or presence of a disease associated with altered mitochondrial function, and therefrom determining the risk or presence of the disease.

Another aspect of the invention provides a method of monitoring a telomere by detecting in a telomeric region the presence of a nucleic acid molecule comprising all or a portion of SEQ ID NO:1 or a complementary portion thereto. In one embodiment, the detected nucleic acid molecule comprises an exmtDNA sequence. In another aspect, the invention provides a method of monitoring a telomere by detecting in a telomeric region the presence of a nucleic acid molecule comprising all or a portion of SEQ ID NO:3 or a complementary portion thereto.

Turning to another aspect, the invention provides a method of identifying an agent suitable for treating a disease associated with altered mitochondrial function, by comparing a ratio *r* from a sample obtained before contacting a biological source with a candidate agent to the ratio *r* from a sample obtained after contacting the biological source with the candidate agent, said ratio *r* calculated using the formula:

$$r = x/(x + y)$$

wherein *x* is the amount of extramitochondrial DNA in a sample, and *y* is the amount of mitochondrial DNA in the sample; and therefrom determining the suitability of said candidate agent for treating a disease associated with altered mitochondrial function. In one embodiment, the biological sample may be a crude buffy coat fraction of whole blood. In another embodiment, the biological sample is treated by heating in water to lyse cells contained in the sample, and then extracting cellular DNA from lysed cells using an aqueous DNA extraction procedure. In another embodiment, the ratio *r* is calculated by contacting a sample containing extramitochondrial DNA and mitochondrial DNA with an oligonucleotide primer having a nucleotide sequence that is complementary to a sequence present in the extramitochondrial DNA and present in the mitochondrial DNA, under conditions and for a time sufficient to allow hybridization of the primer to the extramitochondrial DNA and to the mitochondrial DNA; and detecting hybridization of the primer to the extramitochondrial DNA and to the mitochondrial

DNA, and therefrom quantifying the extramitochondrial DNA and the mitochondrial DNA to calculate the ratio *r*.

In another embodiment of the invention, the ratio *r* is calculated by contacting a sample containing extramitochondrial DNA and mitochondrial DNA with an oligonucleotide primer having a nucleotide sequence that is complementary to a sequence present in the extramitochondrial DNA and present in the mitochondrial DNA, under conditions and for a time sufficient to allow hybridization of the primer to the extramitochondrial DNA and to the mitochondrial DNA; and detecting hybridization and extension of the primer to the extramitochondrial DNA to produce a first product and hybridization and extension of the primer to the mitochondrial DNA to produce a second product distinguishable from the first product, and therefrom quantifying the extramitochondrial DNA and the mitochondrial DNA to calculate the ratio. In another embodiment, the ratio *r* is calculated by contacting a sample containing amplified extramitochondrial DNA and mitochondrial DNA with an oligonucleotide primer having a nucleotide sequence that is complementary to a sequence present in said amplified extramitochondrial DNA and present in said amplified mitochondrial DNA, under conditions and for a time sufficient to allow hybridization of said primer to the extramitochondrial DNA and to the mitochondrial DNA; and detecting hybridization of the primer to the extramitochondrial DNA and to the mitochondrial DNA, and therefrom quantifying the extramitochondrial DNA and the mitochondrial DNA to calculate the ratio *r*.

In yet another embodiment, the ratio *r* is calculated by contacting a sample containing amplified extramitochondrial DNA and mitochondrial DNA with an oligonucleotide primer having a nucleotide sequence that is complementary to a sequence present in the amplified extramitochondrial DNA and present in the amplified mitochondrial DNA, under conditions and for a time sufficient to allow hybridization of said primer to the extramitochondrial DNA and to the mitochondrial DNA; and detecting hybridization and extension of the primer to the extramitochondrial DNA to produce a first product and hybridization and extension of the primer to the mitochondrial DNA to produce a second product distinguishable from said first product,

and therefrom quantifying the extramitochondrial DNA and the mitochondrial DNA to calculate the ratio.

In another embodiment of the invention, comparing the ratio *r* from a sample obtained before contacting a biological source with a candidate agent to the ratio *r* from a sample obtained after contacting the biological source with the candidate agent comprises determination of the presence in the sample of a nucleotide sequence of SEQ ID NO:1 or portion thereof, or a nucleotide sequence of SEQ ID NO:3 or a portion thereof, or an extramitochondrial DNA sequence comprising a nucleic acid sequence that (i) corresponds to at least a portion of SEQ ID NO:2 and (ii) contains at least one nucleotide substitution of Figure 4 at a corresponding nucleotide position, or an extramitochondrial DNA sequence comprising a nucleic acid sequence that (i) corresponds to at least a portion of SEQ ID NO:2 and (ii) contains at least one nucleotide deletion of Figure 4 at a corresponding nucleotide position. In another embodiment, the nucleotide sequence of SEQ ID NO:1 or a portion thereof corresponds to a mitochondrial cytochrome c oxidase encoding sequence of SEQ ID NO:2 or a portion thereof. In another embodiment, the mitochondrial cytochrome c oxidase encoding sequence of SEQ ID NO:2 or a portion thereof is a sequence encoding CO I or a portion thereof, or a sequence encoding CO2 or a portion thereof. In another embodiment, the nucleotide sequence of SEQ ID NO:1 or portion thereof, or the nucleotide sequence of SEQ ID NO:3 or portion thereof corresponds to a mitochondrial ATP synthetase subunit encoding sequence of SEQ ID NO:2 or a portion thereof. In another embodiment, the mitochondrial ATP synthetase subunit encoding sequence of SEQ ID NO:2 or a portion thereof may be a sequence encoding ATP synthetase subunit 6 or a portion thereof, or a sequence encoding ATP synthetase subunit 8 or a portion thereof. In another embodiment, the nucleotide sequence of SEQ ID NO:1 corresponds to a sequence of SEQ ID NO:2 or a portion thereof that may be a sequence encoding a truncated NADH dehydrogenase subunit 1 or a portion thereof, a sequence encoding NADH dehydrogenase subunit 2 or a portion thereof or a sequence encoding truncated CO3 or a portion thereof.

In other embodiments of the invention, the disease associated with altered mitochondrial function may be Alzheimer's Disease, Huntington's Disease, Parkinson's Disease, dystonia, schizophrenia, non-insulin dependent diabetes mellitus, mitochondrial encephalopathy, lactic acidosis, and stroke, myoclonic epilepsy ragged red fiber syndrome, or Leber's hereditary optic neuropathy.

In another aspect, the invention provides a method of identifying an agent suitable for treating a subject suspected of being at risk for having a disease associated with altered mitochondrial function, by determining the apolipoprotein E genotype of the subject; comparing a ratio r in a biological sample obtained from the subject before contacting the sample with a candidate agent to the ratio r in a biological sample obtained from the subject after contacting the sample with a candidate agent, the ratio r calculated using the formula:

$$r = x/(x + y)$$

wherein x is the amount of extramitochondrial DNA in the sample, and y is the amount of mitochondrial DNA in the sample; and therefrom determining the suitability of said candidate agent for treating the disease associated with altered mitochondrial function. In another embodiment, the disease associated with altered mitochondrial function is Alzheimer's disease.

It is another aspect of the invention to provide a method of correlating a ratio r with the suitability of an agent for treating Alzheimer's disease in a subject, by determining a ratio r in a biological sample obtained from the subject, said ratio r calculated using the formula:

$$r = x/(x + y)$$

wherein x is the amount of extramitochondrial DNA in the sample, and y is the amount of mitochondrial DNA in the sample; contacting said subject with a candidate agent and evaluating the subject for alterations in the AD disease state, and therefrom correlating the suitability of the agent for treating AD in the subject with r. In another embodiment, the apolipoprotein E genotype of the subject is determined, and therefrom

the suitability of the agent for treating AD in the subject is correlated with *r* and with the apolipoprotein E genotype.

These and other aspects of the present invention will become evident upon reference to the following detailed description and attached drawings. In addition, various references are set forth herein which describe in more detail certain aspects of this invention, and are therefore incorporated by reference in their entirety.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 depicts the nucleotide sequence of SEQ ID NO:1.

Figure 2 depicts the nucleotide sequence of SEQ ID NO:2.

Figure 3 depicts the nucleotide sequence of SEQ ID NO:3.

Figure 4 depicts human extramitochondrial DNA nucleotide substitutions and deletions relative to the corresponding human mtDNA sequence of SEQ ID NO:2.

Figure 5 shows radiation hybrid chromosomal mapping of an extramitochondrial DNA sequence.

Figure 6 depicts detection of mtDNA and exmtDNA by Southern blot hybridization.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is directed generally to compositions and methods for diagnosing the risk or presence of a disease associated with altered mitochondrial function in a subject, and to compositions and methods for the identification of agents that may be suitable for treating a disease associated with altered mitochondrial function. The invention utilizes a ratio, *r*, that may be useful for pharmacogenomic purposes, for example to stratify patient populations according to the suitability of particular therapeutic agents for use in such populations. The ratio *r* is the ratio of the amount of exmtDNA in a biological sample relative to the sum of the amount of exmtDNA plus mtDNA in the sample. As expressed quantitatively, the ratio *r* may be calculated using the formula:

$$r = x/(x + y)$$

wherein

x is the amount of exmtDNA in a sample, and

y is the amount of mtDNA in the sample.

In various aspects of the invention, as elaborated more fully herein, quantification of x and y provide, through calculation of r, parameters useful in diagnosis of a disease associated with altered mitochondrial function and in screening assays for agents that may be suitable for the treatment of such a disease.

As discussed above, "altered mitochondrial function" may refer to any condition or state, including those that accompany a disease, where any structure or activity that is directly or indirectly related to a mitochondrial function has been changed. Altered mitochondrial function may have its origin in extramitochondrial structures or events as well as in mitochondrial structures or events, in direct interactions between mitochondrial and extramitochondrial genes and/or their gene products, or in structural or functional changes that occur as the result of interactions between intermediates that may be formed as the result of such interactions, including metabolites, catabolites, substrates, precursors, cofactors and the like.

Also as discussed above, altered mitochondrial function may include (but need not be limited to) altered respiratory or metabolic activity in some or all cells of a biological source. For example, markedly impaired ETC activity may be an example of altered mitochondrial function, as may be generation of increased ROS or defective oxidative phosphorylation. As further examples, altered mitochondrial membrane potential, induction of apoptotic pathways and formation of atypical chemical and biochemical crosslinked species within a cell, whether by enzymatic or non-enzymatic mechanisms, may all be regarded as indicative of altered mitochondrial function. Without wishing to be bound by theory, alterations in the ratio r are believed to reflect chemical changes within affected cells that quantitatively influence recoveries of exmtDNA and/or mtDNA. For example, biochemical crosslinking events may result in the formation of DNA aggregates, DNA adducts or other molecular species that affect DNA recoveries following extraction procedures.

According to the present invention, alterations in the ratio *r* as defined above provide a novel and useful parameter for diagnosing the risk or presence of a disease associated with altered mitochondrial function in a subject, and for identifying agents that may be suitable for treating a disease associated with altered mitochondrial function. As discussed above, a number of diseases, including several degenerative diseases, are associated with alterations in mitochondrial function. Further, detection of an appropriate parameter of altered mitochondrial function can provide preclinical evidence for a risk of or predisposition to a disease.

Determination of the ratio *r* involves quantification of exmtDNA (*x*) and mtDNA (*y*) that may be based on strong but not necessarily absolute nucleotide sequence conservation when corresponding portions of mtDNA and exmtDNA are compared, as discussed herein. In most preferred embodiments of the invention, determination of *r* is accomplished by detecting minor nucleotide sequence differences in highly conserved mtDNA and exmtDNA regions, as elaborated below. The invention provides compositions and methods that include the use of nucleic acid molecules, or portions thereof, having nucleotide sequences that are found in the human mtDNA sequence SEQ ID NO:2 (Anderson et al., *Nature 290*:457, 1981) and fragments of SEQ ID NO:2 that are suitable for use as oligonucleotide primers in nucleic acid primer extension or amplification techniques, as hybridization probes for the detection of complementary nucleotide sequences in a sample or for any number of additional uses that are well known to those familiar with the art. ExmtDNA may be nuclear DNA, including chromosomal and non-chromosomal DNA, or non-nuclear extramitochondrial DNA that may be from any subcellular compartment, provided it is not mtDNA.

Nucleic acid sequences within the scope of the invention include isolated DNA and RNA sequences that specifically hybridize under conditions of moderate or high stringency to exmtDNA nucleotide sequences, including exmtDNA sequences disclosed herein or fragments thereof, and their complements. As used herein, conditions of moderate stringency, as known to those having ordinary skill in the art, and as defined by Sambrook et al., *Molecular Cloning: A Laboratory Manual*, 2nd Ed.

Vol. 1, pp. 1.101-104. Cold Spring Harbor Laboratory Press (1989), include use of a prewashing solution for the nitrocellulose filters 5X SSC, 0.5% SDS, 1.0 mM EDTA (pH 8.0), hybridization conditions of 50% formamide, 6X SSC at 42°C (or other similar hybridization solution), and washing conditions of about 50-60°C, 0.5X SSC, 0.1% SDS. Conditions of high stringency are defined as hybridization conditions as above, and with washing at 60-68°C, 0.2X SSC, 0.1% SDS. In other embodiments, hybridization to an exmtDNA nucleotide sequence may be at normal stringency, which is approximately 25-30°C below Tm of the native duplex (*e.g.*, 5X SSPE, 0.5% SDS, 5X Denhardt's solution, 50% formamide, at 42°C or equivalent conditions), at low stringency hybridizations, which utilize conditions approximately 40°C below Tm, or at high stringency hybridizations, which utilize conditions approximately 10°C below Tm. The skilled artisan will recognize that the temperature, salt concentration, and chaotrope composition of hybridization and wash solutions may be adjusted as necessary according to factors such as the length and nucleotide base composition of the probe. (*See also, e.g.*, Ausubel et al., *Current Protocols in Molecular Biology*, Greene Publishing, 1987.)

An "isolated nucleic acid molecule" refers to a polynucleotide molecule in the form of a separate fragment or as a component of a larger nucleic acid construct, that has been separated from its source cell (including the chromosome it normally resides in) at least once, preferably in a substantially pure form. Isolated nucleic acids may be nucleic acids having particular disclosed nucleotide sequences or may be regions, portions or fragments thereof. Those having ordinary skill in the art are able to prepare isolated nucleic acids having the complete nucleotide sequence, or the sequence of any portion of a particular isolated nucleic acid molecule, when provided with the appropriate nucleic acid sequence information as disclosed herein. Nucleic acid molecules may be comprised of a wide variety of nucleotides, including DNA, RNA, nucleotide analogues such as phosphorothioates or peptide nucleic acids, or other analogues with which those skilled in the art will be familiar, or some combination of these.

The present invention, as described herein, provides exmtDNA sequences and isolated exmtDNA nucleic acid molecules. exmtDNA may be isolated from genomic DNA, typically by first generating an appropriate DNA library through techniques for constructing libraries that are known in the art (*see* Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Press, 1989) or purchased from commercial sources (*e.g.*, Clontech, Palo Alto, California). Briefly, genomic DNA libraries can be constructed in chromosomal vectors, such as YACs (yeast artificial chromosomes), bacteriophage vectors, such as pBeloBAC11, λEMBL3, λgt10, cosmids, or plasmids. Alternatively, isolated exmtDNA may be prepared by preferentially amplifying exmtDNA sequences present in biological samples using, for example, DNA amplification methodologies such as PCR or other amplification techniques that are well known in the art, with suitable oligonucleotide primers complementary to exmtDNA sequences as disclosed herein.

In one embodiment, known mtDNA sequences derived from SEQ ID NO:2 (Anderson et al., *Nature 290*:457, 1981) may be utilized to design oligonucleotide hybridization probes suitable for screening genomic libraries. Preferably, such oligonucleotide probes are 18-30 bases in length and have sequences that, under the hybridization conditions selected, hybridize to complementary exmtDNA sequences lacking nucleotide substitutions, insertions or deletions ("mutations") relative to the corresponding region of the mtDNA sequence of SEQ ID NO:2.

Portions of an exmtDNA sequence and the mtDNA sequence of SEQ ID NO:2 are regarded as "corresponding" nucleic acid sequences, regions, fragments or the like, based on the convention for numbering mtDNA nucleic acid positions according to SEQ ID NO:2 (Anderson et al., *Nature 290*:457, 1981), wherein an exmtDNA sequence is aligned with the mtDNA sequence of SEQ ID NO:2 such that at least 70%, preferably at least 80% and more preferably at least 90% of the nucleotides in a given sequence of at least 20 consecutive nucleotides of a sequence are identical. In certain preferred embodiments, an exmtDNA sequence is greater than 95% identical to a corresponding mtDNA sequence. In certain particularly preferred embodiments, an exmtDNA sequence is identical to a corresponding mtDNA sequence. Those oligonucleotide

probes having sequences that are identical in corresponding regions of mtDNA and exmtDNA may be identified and selected following hybridization target DNA sequence analysis. to verify the absence of mutations in the target exmtDNA sequence relative to the primer mtDNA-derived sequence.

To facilitate hybridization detection, the oligonucleotide may be conveniently labeled, generally at the 5' end, with a reporter molecule, such as a radionuclide, *e.g.*, ^{32}P, enzymatic label, protein label, fluorescent label, biotin or other suitable labeling moieties known in the art. Such libraries are then generally plated as phage or colonies, depending upon the vector used. Subsequently, a plate replica to which the colonies or phage have been transferred, such as a nitrocellulose or nylon membrane or the like, is probed to identify candidate clones that contain the exmtDNA sequence. Such candidates may be verified as containing exmtDNA by any of various means including, for example, DNA sequence analysis or hybridization with a second, non-overlapping probe selected as described above to hybridize with target exmtDNA sequences lacking nucleotide substitutions, deletions or insertions relative to the corresponding portion of the mtDNA sequence of SEQ ID NO:2.

Once a library is identified as containing exmtDNA, the exmtDNA can be isolated by amplification. Briefly, when using genomic library DNA as a template, amplification primers are designed based upon known mtDNA sequences (SEQ ID NO:2) and primer "walking" is used to select primers that anneal to exmtDNA regions that are identical to mtDNA sequences. The primers preferably have a GC content of about 50% and contain restriction sites to facilitate cloning. Primers do not have self-complementary sequences, nor do they contain complementary sequences at their 3' end (to prevent primer-dimer formation). The primers are annealed to genomic DNA and sufficient amplification cycles are performed to yield a product readily visualized by gel electrophoresis and staining. The amplified fragment is purified and inserted into a vector, such as λgt10 or pBS(M13+), and propagated. Confirmation of the nature of the fragment is obtained by DNA sequence analysis.

As an example of detection of mtDNA-like sequences in a DNA library, an oligonucleotide having a nucleotide sequence present in a portion of any human

mtDNA gene, preferably one of the human mtDNA encoded genes NADH dehydrogenase subunit 1 (ND1), NADH dehydrogenase subunit 2 (ND2), NADH dehydrogenase subunit 3 (ND3), NADH dehydrogenase subunit 4 (ND4), NADH dehydrogenase subunit 4L (ND4L), NADH dehydrogenase subunit 5 (ND5), NADH dehydrogenase subunit 6 (ND6), $tRNA^{Lys}$, $tRNA^{Ile}$, $tRNA^{Gln}$, $tRNA^{Met}$, $tRNA^{Cys}$, $tRNA^{Tyr}$, $tRNA^{Asp}$ or cytochrome c oxidase 3 (CO3) and more preferably one of the human mtDNA encoded genes CO1, CO2, ATPase 8 or ATPase 6, may be labeled and used as a probe on a human genomic DNA library. An initial hybridization at normal stringency may yield candidate clones or fragments. If no hybridization is initially observed, varying degrees of stringency may be used. (*See* Sambrook et al., Ausubel et al., *supra*, and other well-known sources for stringency conditions.)

Where it is advantageous to use oligonucleotide primers according to the present invention, such primers may be 10-60 nucleotides in length, preferably 15-35 nucleotides and still more preferably 18-30 nucleotides in length. Primers as described above for use in isolating exmtDNA from genomic DNA may also be useful in the present invention for quantifying mtDNA and exmtDNA by any of a variety of techniques well known in the art for determining the amount of specific nucleic acid target sequences present in a sample based on specific hybridization of a primer to the target sequence. Optionally, in certain of these techniques, hybridization precedes nucleotide polymerase catalyzed extension of the primer using the strand containing the target sequence as a template, and/or ligation of oligonucleotides hybridized to adjacent target sequences, and embodiments of the invention using primer extension are particularly preferred. For examples of references on such quantitative detection techniques, including those that may be used to detect nucleotide insertions, substitutions or deletions in a portion of an exmtDNA sequence site near an oligonucleotide primer target hybridization site that corresponds to a portion of the mtDNA sequence of SEQ ID NO:2, and further including those that involve primer extension, see U.S. 5,760,205 and the references cited therein, all of which are hereby incorporated by reference, and see also, for example, Botstein et al. (*Am. J. Hum. Gen.* *32*:314, 1980), Gibbs et al. (*Nucl. Ac. Res.* *17*:2437, 1989), Newton et al. (*Nucl. Ac. Res.*

17:2503, 1989), Grossman et al. (*Nucl. Ac. Res. 22*:4527, 1994), and Saiki et al. (*Proc. Nat. Acad. Sci. 86*:6230, 1989), all of which are hereby incorporated by reference. A particularly useful method for this purpose is the primer extension assay disclosed by Fahy et al. (*Nucl. Acids Res. 25*:3102, 1997) and by Ghosh et al. (*Am. J. Hum. Genet. 58*:325, 1996), both of which references are hereby incorporated in their entireties, as is Krook et al. (*Hum. Molec. Genet. 1*:391, 1995) which teaches modification of primer extension reactions to detect multiple nucleotide substitutions, insertions, deletions or other mutations. Other examples of useful techniques for quantifying the presence of specific nucleic acid target sequences in a sample include but need not be limited to labeled probe hybridization to the target nucleic acid sequences with or without first partially separating target nucleic acids from other nucleic acids present in the sample.

Examples of other useful techniques for determining the amount of specific nucleic acid target sequences present in a sample based on specific hybridization of a primer to the target sequence include specific amplification of target nucleic acid sequences and quantification of amplification products, including but not limited to polymerase chain reaction (PCR, Gibbs et al., *Nucl. Ac. Res. 17*:2437, 1989), transcriptional amplification systems, strand displacement amplification and self-sustained sequence replication (3SR, Ghosh et al, in Molecular Methods for Virus Detection, 1995 Academic Press, NY, pp. 287-314), the cited references for which are hereby incorporated in their entireties. Examples of other useful techniques include ligase chain reaction, single stranded conformational polymorphism analysis, Q-beta replicase assay, restriction fragment length polymorphism (RFLP, Botstein et al., *Am. J. Hum. Gen. 32*:314, 1980) analysis and cycled probe technology, as well as other suitable methods that will be known to those familiar with the art.

In a particularly preferred embodiment of the invention, primer extension is used to quantify exmtDNA and mtDNA present in a biological sample. (Ghosh et al., *Am. J. Hum. Genet. 58*:325, 1996) This embodiment may offer certain advantages by permitting both exmtDNA and mtDNA to be simultaneously quantified using a single oligonucleotide primer capable of hybridizing to a complementary nucleic acid target sequence that is present in a defined region of mtDNA and in a corresponding region of

a exmtDNA sequence. Without wishing to be bound by theory, the use of a single primer for quantification of exmtDNA and of mtDNA is believed to avoid uncertainties associated with potential disparities in the relative hybridization properties of multiple primers and may offer other advantages. Where such a target sequence is situated adjacent to an exmtDNA nucleotide sequence position that is a nucleotide substitution, insertion or deletion relative to the corresponding mtDNA sequence position, primer extension assays may be designed such that oligonucleotide extension products of primers hybridizing to mtDNA are of different lengths than oligonucleotide extension products of primers hybridizing to exmtDNA. Accordingly, the amount of exmtDNA in a sample and the amount of mtDNA in the sample may be determined by quantification of distinct extension products that are separable on the basis of sequence length or molecular mass, for purposes of calculating the ratio *r* as described above.

Sequence length or molecular mass of primer extension assay products may be determined using any known method for characterizing the size of nucleic acid sequences with which those skilled in the art are familiar. In a preferred embodiment, primer extension products are characterized by gel electrophoresis. In another preferred embodiment, primer extension products are characterized by mass spectrometry (MS), which may further include matrix assisted laser desorption ionization/ time of flight (MALDI-TOF) analysis or other MS techniques known to those having skill in the art. See, for example, U.S. 5,622,824, U.S. 5,605,798 and U.S. 5,547,835, all of which are hereby incorporated by reference in their entireties. In another preferred embodiment, primer extension products are characterized by liquid or gas chromatography, which may further include high performance liquid chromatography (HPLC), gas chromatography-mass spectrometry (GC-MS) or other well known chromatographic methodologies.

Any exmtDNA sequence or portion of an exmtDNA sequence that corresponds to the human mtDNA sequence of SEQ ID NO:2 or a portion thereof or several portions thereof may be useful in this embodiment of the invention. Examples of human exmtDNA sequences that are useful in this and other embodiments of the invention are disclosed in SEQ ID NO:1, in SEQ ID NO:3 and in SEQ ID NO:4.